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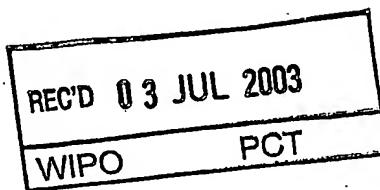
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**Technical field**

The present invention relates to a method for identifying compounds that will inhibit DNA transcription, and to compounds identified by this method. Furthermore, it relates to a compound that is useful in the treatment of hyperproliferative disorders. The compound may also be used as a tool in the field of cell and molecular biology.

**Background of the invention**

A large number of diseases are caused by an increase in cell proliferation. The most prominent of such diseases is cancer. As is well known, cancer is a major cause of suffering and death around the world and new therapies are continuously sought. A drug with antiproliferative effect would desirably be directed against a target or process which is vital for the proliferating cells. Several such targets or processes may be envisaged, such as a DNA polymerase for inhibiting replication, or an RNA polymerase for inhibiting transcription. A central activity in cells that are undergoing rapid cell proliferation is the transcription of DNA to RNA. This forms the basis of an opportunity to treat diseases caused by an increased cell proliferation. The transcription-inhibiting compound Actinomycin D has been used as a cytostatic drug in cancer treatment, but carries with it non desirable side effects. Consequently, there is a need for other anti-hyperproliferative drugs, to be used either on its own or in combination therapies together with other drugs.

Several treatments have previously been used for psoriasis; these include the administration of compounds such as steroids (cortisone), anthralin, Vitamin D, retinoids, coal tar, light therapy, Cyclosporine, Methotrexate, Psoralen, or combinations thereof.

There are many fast and effective methods to introduce transcriptionally active DNA into cells, but options for delivering functional proteins into cells are limited. New research and commercially available products offer another approach: protein transduction.

5 The mammalian cell membrane is refractory to most proteins and peptides. Until recently, the most widely used methods to introduce antibodies, peptides, or other membrane-impermeable molecules into cells were microinjection and electroporation- both invasive techniques that disrupt the cell membrane.

10 Microinjection involves loading or transferring a dissolved substance into a living cell using the microscopic tip of a glass capillary. The technique requires technical skill and can only be performed one cell at a time. Electroporation uses high-voltage pulses to produce transient pores through which proteins can enter. While this technique can be applied to many cells at once, it is toxic and non-specific—that is, anything can enter or exit the cell once the membrane is disrupted.

### Summary of the invention

15 The inventors have identified the interaction between actin and Hrp65 as, surprisingly, being important for the transcription of genes. This opens up the possibility to use actin and Hrp65 in an assay for identifying compounds that will inhibit said interaction. Using such an assay, the inventors have identified a novel peptide which has the unexpected effect of inhibiting transcription. Accordingly, the present invention relates to a compound and its analogues with an inhibitory effect on transcription. Being inhibitors of transcription, the compound or analogues thereof according to the invention, are useful in the treatment of diseases caused by hyperproliferating cells. Examples of such diseases include various types of cancers, and also non-cancer like diseases such as rheumatoid arthritis or psoriasis. Another 20 aspect of the invention is to use the identified inhibitors as tools used in cell and molecular biology in experiments where it is necessary to inhibit transcription, or in protein transduction methods.

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### Detailed description of the invention

The protein actin is a nuclear component. Early observations based on microinjection of either anti-actin antibodies or actin-binding proteins into the nuclei of living 5 amphibian oocytes suggested that nuclear actin is involved in transcription of protein-coding genes. In the process of gaining further insight into the function(s) of nuclear actin, the inventors sought to identify proteins which bind to actin in the cell nucleus. Affinity chromatography experiments were performed using nuclear extracts of *Chironomus tentans*. A protein of approximately 65 kDa was identified and denoted 10 Hrp65 (amino acid sequence shown in SEQ ID NO 4). Three nuclear proteins in humans share homology with Hrp65: PSF, p54nrb, and PSP1. Multiple functions have been attributed to these proteins, from splicing to transcription regulation to retention 15 of RNA in the nucleus and it is not known whether these proteins bind actin.

The inventors subsequently showed that actin is able to bind to the isoforms Hrp65-1 and Hrp65-2, but not Hrp65-3. Since the Hrp65 isoforms are identical from amino acid 20 residue 1 to 499, their differential behaviour in the actin-binding assay suggested that the variable C-terminal sequences were involved in actin binding. To test this hypothesis, a peptide termed 65-2CTS (hereinafter also termed TIP (as in transcription inhibiting peptide), sequence shown in SEQ ID NO 1) containing the last 15 C-terminal amino acid residues of Hrp65-2 was chemically synthesized and conjugated to a carrier, keyhole limpet hemocyanin (KLH). Actin-Hrp65 binding assays were then 25 performed in the presence of saturating amounts of either KLH-65-2CTS or KLH alone. KLH65-2CTS was able to compete specifically for binding of actin to Hrp65-2. These data indicate that actin binds specifically to the Hrp65-2 isoform and strongly suggest that the C-terminus of Hrp65-2 is implicated in this interaction.

30 The identification of Hrp65 as an actin binding protein, together with the further results presented herein, i. e. the finding that disruption of the actin/Hrp65 interaction causes inhibition of transcription, demonstrates that the actin/Hrp65 system would be a useful tool for identifying compounds that may be used as drugs for treating hyperproliferative diseases, such as cancer. Consequently, one aspect of the present

invention is an assay which can be used for identifying compounds that are capable of inhibiting, preventing or in other ways disturbing, blocking or abolishing the interaction between an actin molecule and an Hrp65 molecule. As described herein, blocking this interaction has the effect of inhibiting transcription of DNA into RNA.

5 The method according to the invention may be adapted to suit a high through-put screening format, but other variants are also envisaged.

A general assay according to the invention may comprise the following steps;

- (a) providing an Hrp65 molecule,
- 10 (b) adding an actin molecule to said Hrp65 molecule, thereby forming a complex between actin and Hrp65,
- (c) adding a test compound to said complex,
- (d) determining the effect of said test compound on said complex.

15 The order of dispensing is not crucial for the assay, it is therefore possible to start with the actin molecule, followed by addition of the Hrp65 molecule. The test compound may be added after the complex has formed, or it may be added together with one of the protein molecules. It is well within the capacity of the skilled person to determine if a test compound has an effect on the interaction between Hrp65 and actin. Such methods may include mass spectroscopy, gel filtration, CD spectroscopy, in which one determines the molecular mass of a protein. One could also use fluorescence resonance 20 energy transfer (FRET) wherein one labels the two proteins with different fluorophores with different absorption spectra and overlapping donor emission/acceptor absorption-spectra. The method is described in Wu and Brand, Analytical Biochem, 218, 1-13, 1994, Watson BS, Hazlett TL, Eccleston JF, Davis C, 25 Jameson DM, Johnson AE. Biochemistry 34, 7904-7912 (1995), and in Moens PD, Yee DJ, dos Remedios CG. Biochemistry 33, 13102-13108 (1994)

30 An assay to identify compounds which will inhibit the interaction may comprise the following steps; (a) conjugating Hrp65 to a solid support, such as a bead, resin, surface, or in a well in a multiwell plate, (b) removing any non-conjugated Hrp65 by

washing in a suitable buffer, (c) blocking non-conjugated sites on the solid support by adding another protein (e.g. bovine serum albumin, or a blocking mixture such as milk powder), (d) adding labeled (e.g. radioactively, by e.g.  $^{35}\text{S}$ , or fluorescently, by e.g. FITC or fluorescein-5-maleimide) actin to the conjugated Hrp65, (e) washing away unbound actin, by washing with a suitable buffer, (f) optionally calculating the amount of formed actin/Hrp65 complexes by measuring the amount of bound labeled molecule, (g) adding a test compound to the formed complexes, (h) collecting the displaced actin and measuring either the amount of displaced actin or the amount of actin remain in complex with Hrp65 (e.g. by scintillation counting or measuring fluorescence, or quantifying the amount on an SDS-polyacrylamide gel) the amount of displaced actin, and (f) comparing the amount of displaced actin in the presence of test compound with the amount of unbound actin in the absence of test compound. It may be recommendable to use as control a mock sample containing only buffer (i. e. no test compound). The method could just as well be performed by exchanging actin for Hrp65 and Hrp65 for actin, in which case actin is conjugated to the solid support and labeled Hrp65 is used. A compound which prevents more than half of the actin molecules to bind to Hrp65 at a concentration lower than  $100\mu\text{M}$ , or preferably  $10\mu\text{M}$ , or more preferably  $1\mu\text{M}$ , is considered to be an inhibitor of the actin/Hrp65 interaction and may therefore be useful in treating antiproliferative diseases. FITC is fluorescein isothiocyanate, and is obtainable from Molecular Probes, or from Sigma. It should be noted that either Hrp65 or actin could be conjugated to the solid support. In the case of conjugating actin to the solid support, labeled Hrp65 is then added in step (d). To prevent non-specific binding of the labeled protein to the solid support, sites on the solid support which has not been conjugated to the actin or Hrp65, respectively, is preferably blocked by adding another protein or blocking mixture, such as bovine serum albumin or milk powder. Another way to detect binding between Hrp65 and actin is by immunochemical detection. In this case, one does not need to label the proteins, but the presence of bound protein after step (h) is detected by the use of an antibody against the non-conjugated protein. For example, if actin is conjugated to the solid carrier, an antibody directed against Hrp65 may be used to detect the presence of Hrp65 after washing off unbound protein. Actin may be obtained according to the

method disclosed in Zechel, K., (1980). Hrp65 can be obtained according to the procedure described herein (Example 5). Alternatively, recombinant Hrp65 can be produced in bacteria with a histidine tag and affinity purified on Ni-agarose beads according to standard procedures. It should be noted that functionally equivalent forms of the two proteins in the assay may be used. Therefore, analogues, such as 5 homologous proteins may be used. The only provision is that the proteins can interact in essentially the same way as the proteins that are used in the work described herein. Test compounds may be any compound, including peptides, polypeptides, organic or inorganic molecules.

10 The inventors have used a variant of the above mentioned method, in which both Hrp65 and actin have been radioactively labeled, to be able to detect, by autoradiography, both proteins on a sodium dodecyl sulphate-polyacrylamide (SDS-PAGE) gel. The results show that actin is able to directly bind to Hrp65-2 (SEQ ID 15 NO 4).

20 By performing the method according to the invention, it is possible to identify compounds that are capable of inhibiting the interaction between actin and Hrp65. Accordingly, such compounds are also within the scope of the invention. By carrying 25 out the method according to the invention, the inventors have identified two novel compounds, shown in SEQ ID NO 1 and SEQ ID NO 3, capable of inhibiting the interaction between actin and Hrp65. These compounds are also capable of inhibiting transcription and thus they are expected to inhibit cell-proliferation, and can be used in the field of medicine. These compounds are internalized by living cells and transported to the cell nucleus. The results presented herein show that a peptide which comprises 30 the amino acid sequence CPYVNQRPK is capable of inhibiting the interaction between actin and Hrp65, but also that other, longer fragments may be useful. The inventors have shown that a polypeptide in which the amino acids in position 7 and 10 of SEQ ID NO 5 (and 12 and 14 in SEQ ID NO 6) are basic, has TIP-activity. It is believed that basic amino acids in said positions are necessary for activity, and consequently, the amino acids in positions 7 and 10, denoted by an X may be any

5 amino acid, but the basic amino acids arginine and lysine are preferred, or most preferably, the amino acids are Q at position 7 and K at position 10. Consequently, one aspect of the invention is a compound comprising the sequence according to SEQ ID NO 5, further comprising the variants mentioned above. Also included in the present invention is a compound comprising the sequence CPYVNQXPQXAXYXNG (SEQ ID NO 6) in which X may be any amino acid, preferably any of the basic amino acids arginine or lysine or it may also be alanine.

10 In one aspect, the invention is a compound chosen from the group comprising; a polypeptide according to SEQ ID NO 1, SEQ ID NO 3, SEQ ID NO 5, or SEQ ID NO 6, analogues or oligomers thereof, fragments thereof, or oligomers thereof. The compound may be a compound having at least 80% similarity, preferably at least 90% similarity, more preferably at least 95%, further more preferably at least 96%, even more preferably at least 97%, or most preferably at least 98% similarity to the 15 polypeptide shown in SEQ ID NO 1, SEQ ID NO 3, SEQ ID NO 5, OR SEQ ID NO 6. More preferably, a compound according to the invention will have at least 80% identity, preferably at least 90% identity, more preferably at least 95%, further more preferably at least 96%, even more preferably at least 97%, or most preferably at least 98%, identity to the polypeptide shown in SEQ ID NO 1, SEQ ID NO 3, SEQ ID NO 20 5, OR SEQ ID NO 6.

In another embodiment, the compounds according to the invention are meant for medical use.

25 In a further embodiment, the compounds according to the invention are used for manufacturing a medicament which is to be used in treating a mammal suffering from a disease caused by hyperproliferative cells. Such diseases are exemplified by melanoma, non-small-cell lung cancer, small-cell lung cancer, lung cancer, hepatocarcinoma, retioblastoma, astrocytoma, glioblastoma, leukemia, neuroblastoma, 30 cancer in the gum, tongue, head, neck, breast, pancreas, prostate, kidney, bone, testicle, ovary, mesothelia, cervix, gastrointestinal tract, lymphoma, brain, colon,

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sarcoma, bladder, rheumatoid arthritis, inflammatory bowel disease, osteoarthritis, leiomyomas, adenomas, lipomas, hemangiomas, fibromas, vascular occlusion, retenosis, atherosclerosis, pre-neoplastic lesions, adenomatous hyperplasia, prostatic intraepithelial neoplasia, carcinoma in situ, oral hairy leukoplakia, or psoriasis.

5 The compounds according to the invention will be administered to patients suffering from the above mentioned diseases such that these patients are conferred a therapeutic benefit as a result of the treatment. The term "therapeutic benefit" used throughout this application refers to anything that promotes or enhances the well-being of the patient with respect to the medical treatment of his hyperproliferative disease. A list of 10 nonexhaustive examples of this includes a reduction of the symptoms of the disease, extension of the patient's life by any period of time; decrease or delay in the neoplastic development of the disease; decrease in hyperproliferation, reduction in tumor growth, delay of metastasis, reduction in the proliferation rate of a cancer cell, tumor cell, or 15 any other hyperproliferative cell; and a decrease in pain to the patients that can be attributed to the patient's condition.

20 In yet another embodiment, the compounds according to the invention will be used in a pharmaceutical composition together with pharmaceutically acceptable excipients and additives. The compositions may be formulated according to conventional pharmaceutical practice, see, e.g., "Remington: The science and practice of pharmacy" 20<sup>th</sup> ed. Mack Publishing, Easton PA, 2000 ISBN 0-912734-04-3 and "Encyclopedia 25 of Pharmaceutical Technology", edited by Swarbrick, J. & J. C. Boylan, Marcel Dekker, Inc., New York, 1988 ISBN 0-8247-2800-9.

30 For psoriasis, weaker preparations should be used on more sensitive areas of the body such as the genitals, groin, and face. Stronger preparations will usually be needed to control lesions on the scalp, elbow, knees, palms and soles, and parts of the torso and may need to be applied under dressings. These must be used cautiously and with the dermatologist's instruction.

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The compounds according to the invention and their physiologically tolerable salts and derivatives can be administered according to the invention to animals, preferably to mammals, and in particular to humans, as pharmaceuticals for therapy or prophylaxis.

5 They can be administered *per se*, in mixtures with one another or in the form of pharmaceutical preparations which permit enteral or parenteral administration and which as active constituent contain an efficacious dose of at least one compound according to the invention and/or its physiologically tolerable salts and derivatives in addition to customary pharmaceutically excipients and/or additives.

10 The pharmaceuticals can be administered systemically or locally. They can be administered, for example, in the form of pills, tablets, film-coated tablets, sugar-coated tablets, granules, hard and soft gelatin capsules, powders, solutions, syrups, emulsions, suspensions or in other pharmaceutical forms. However, administration can also be carried out vaginally or rectally, for example in the form of suppositories, or 15 parenterally or by implantation, for example in the form of injection solutions or infusion solutions, microcapsules or rods, or topically or percutaneously, for example in the form of ointments, solutions or tinctures, or in another way, for example in the form of nasal sprays or aerosol mixtures or as inhalable dry powder preparations. If 20 solutions are parenterally administered they can be administered, for example, intravenously, intramuscularly, subcutaneously, intraarticularly, intrasynovially or in another manner, e.g. by inhalation of wet aerosols or dry powder preparations.

25 The pharmaceutical preparations according to the invention are prepared in a manner known *per se*, it being possible to use pharmaceutically inert inorganic and/or organic excipients in addition to the compound(s) according to the invention and/or its/their physiologically tolerable salts and derivatives. For the preparation of pills, tablets, sugar-coated tablets and hard gelatin capsules, it is possible to use, for example, lactose, cornstarch or derivatives thereof, talc, stearic acid or its salts etc. Excipients for soft gelatin capsules and suppositories are, for example, fats, waxes, semisolid and 30 liquid polyols, polyethylene glycols, natural or hardened oils etc. Suitable excipients for the preparation of solutions, for example injection solutions, or of emulsions or

5 syrups are, for example, water, alcohols, glycerol, diols, polyols, sucrose, invert sugar, glucose, vegetable oils etc. Suitable excipients for microcapsules, implants or rods are, for example, copolymers of glycolic acid and lactic acid. The pharmaceutical preparations normally contain approximately 0.5 to 90% by weight of the compounds according to the invention and/or their physiologically tolerable salts and derivatives.

10 In addition to the active compounds and excipients, the pharmaceutical preparations can additionally contain auxiliaries or additives, such as, for example, fillers, disintegrants, binders, lubricants, wetting agents, stabilizers, emulsifiers, preservatives, sweeteners, colorants, flavorings or aromatizers, thickeners, diluents, buffer substances, solvents or solubilizers, means for achieving a depot effect, salts for altering the osmotic pressure, coating agents or antioxidants. They can also contain 15 two or more compounds according to the invention and/or their physiologically tolerable salts and derivatives. Furthermore, they can also contain one or more other therapeutically or prophylactically active substances in addition to at least one compound according to the invention and/or its physiologically tolerable salts and derivatives. The pharmaceutical preparations normally contain 0.2 to 500 mg, preferably 1 to 100 mg, of active compound according to the invention and/or its physiologically tolerable salts and derivatives per dose.

20 If the compounds according to the invention or pharmaceutical preparations containing them are administered as aerosols, for example as nasal aerosols or by wet aerosols or dry powder inhalation, this can be effected, for example, using a spray, an atomizer, a pump atomizer, an inhalation apparatus, a metered inhaler or a dry powder inhaler, respectively. Pharmaceutical forms for administration of the compounds according to 25 the invention as an aerosol can be prepared by the process well known to the person skilled in the art. For their preparation, for example, solutions or dispersions of the compounds according to the invention in water, water-alcohol mixtures or suitable saline solutions using customary additives, for example benzyl alcohol or other suitable preservatives, absorption enhancers for increasing the bioavailability, solubilizers, dispersants and others, and, if appropriate, customary propellants, for 30

example chlorofluorohydrocarbons and/or fluorohydrocarbons are suitable, whereas dry powder preparations of the compounds according to the invention and/or their physiologically tolerable salts may be obtained by freeze drying or preferably spray drying aqueous solutions of the compounds according to the invention and/or their physiologically tolerable salts and of suitable water soluble additives, such as sugars or sugar derivatives and amino acids.

The dose when using the compounds according to the invention can vary within wide limits, and as customary it is to be tailored to the individual conditions in each 5 individual case, as is known to the physician. It depends, for example, on the nature and severity of the disease to be treated, on the compound employed or whether an acute or chronic disease state is treated or prophylaxis is conducted or on whether further active compounds are administered in addition to the compounds according to 10 the invention. In general, in the case of oral administration, a daily dose of approximately 0.01 to 100 mg/kg, preferably 0.1 to 10 mg/kg, in particular 0.3 to 2 15 mg/kg (in each case per kg of body weight) is appropriate in an adult to achieve effective results. In the case of intravenous administration, the daily dose is in general approximately 0.01 to 50 mg/kg, preferably 0.01 to 10 mg/kg of body weight. In 20 particular when relatively large amounts are administered, the daily dose can be divided into a number, for example 2, 3 or 4, of part administrations. If appropriate, depending on individual behavior, it may be necessary to deviate upward or downward from the indicated daily dose.

Furthermore, the compounds according to the invention and their salts according to the 25 present invention can be used as intermediates for the preparation of other compounds, in particular of other pharmaceutical active compounds which are obtainable from compounds according to the invention, for example, by modification or introduction of radicals or functional groups, for example by esterification, reduction, oxidation or other conversions of functional groups.

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The peptide analogues according to the present invention thus found can on the one hand be used directly as therapeutic agent, but they can also form the basis for related structures, which are also suitable for use as therapeutic agent for treating diseases relating to an increased cell proliferation.

5 An effective amount of the pharmaceutical composition, generally, is defined as that amount sufficient to detectably and repeatedly to ameliorate, reduce, minimize or limit the extent of the disease or its symptoms. More rigorous definitions may apply, including elimination, eradication or cure of disease.

10 To kill cells, inhibit cell growth, inhibit metastasis, decrease tumor or tissue size and otherwise reverse or reduce the malignant phenotype of tumor cells, using the compounds and compositions of the present invention, one would generally contact a hyperproliferative cell with the compound(s) or composition(s). The routes of administration will vary, naturally, with the location and nature of the lesion, and include, e.g. intradermal, transdermal, parenteral, intravenous, intramuscular, 15 intranasal, subcutaneous, percutaneous, intratracheal, intraperitoneal, intratumoral, perfusion, lavage, direct injection, and oral administration and formulation.

20 Intratumoral injection, or injection into the tumor vasculature is specifically contemplated for discrete, solid, accessible tumors. Local, regional or systemic administration also may be appropriate. For tumors of >4 cm, the volume to be administered will be about 4-10 ml (preferably 10 ml), while for tumors of <4 cm, a volume of about 1-3 ml will be used (preferably 3 ml). Multiple injections delivered as 25 single dose comprise about 0.1 to about 0.5 ml volumes. The hyperproliferating cells may be contacted by administering multiple injections to the tumor, spaced at approximately 1-cm intervals.

30 In the case of surgical intervention, the present invention may be used preoperatively, to render an inoperable tumor subject to resection. Alternatively, the present invention may be used at the time of surgery, and/or thereafter, to treat residual or metastatic

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disease. For example, a resected tumor bed may be injected or perfused with a formulation comprising the compounds according to the invention. The perfusion may be continued post-resection, for example, by leaving a catheter implanted at the site of the surgery. Periodic post-surgical treatment also is envisioned.

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Continuous administration also may be applied where appropriate, for example, where a tumor is excised and the tumor bed is treated to eliminate residual, microscopic disease. Delivery via syringe or catheterization is preferred. Such continuous perfusion may take place for a period from about 1-2 hours, to about 2-6 hours, to about 6-12 hours, to about 12-24 hours, to about 1-2 days, to about 1-2 weeks or longer following the initiation of treatment. Generally, the dose of the therapeutic composition via continuous perfusion will be equivalent to that given by a single or multiple injections, adjusted over a period of time during which the perfusion occurs. It is further contemplated that limb perfusion may be used to administer therapeutic compositions of the present invention, particularly in the treatment of melanomas and sarcomas.

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Treatment regimens may vary as well, and often depend on tumor type, tumor location, disease progression, and health and age of the patient. Obviously, certain types of tumor will require more aggressive treatment, while at the same time, certain patients cannot tolerate more taxing protocols. The clinician will be best suited to 20 make such decisions based on the known efficacy and toxicity (if any) of the therapeutic formulations.

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In certain embodiments, the tumor being treated may not, at least initially, be resectable. Treatments with therapeutic viral constructs may increase the resectability of the tumor due to shrinkage at the margins or by elimination of certain particularly invasive portions. Following treatments, resection may be possible. Additional treatments subsequent to resection will serve to eliminate microscopic residual disease 30 at the tumor site.

A typical course of treatment, for a primary tumor or a post-excision tumor bed, will involve multiple doses. Typical primary tumor treatment involves a 6-dose application over a two-week period. The two-week regimen may be repeated one, two, three, four, five, six or more times. During a course of treatment, the need to complete the planned dosings may be re-evaluated.

In another aspect, the invention relates to the use of the compounds according to the invention as a tool within the fields of cell and molecular biology. An experimental approach used in cell biology research is to disturb cellular processes and to analyze the effect(s) of the disturbance on the cell function(s). For example, in studies of gene expression it can be interesting to inhibit transcription in order to be able to analyze cellular processes in cells that are no longer able to produce RNA. Several commercially available drugs can be used to inhibit transcription. Actinomycin D (also used as a cytostatic drug in cancer treatment), alpha-amanitin, and 5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole (DRB) are the most commonly used. Virtually all drugs can have secondary effects on living cells, and to discriminate between secondary effects and direct effects due to transcription inhibition, it is usually recommended to carry out parallel experiments with different inhibitors. The mechanism of action of each drug is specific, and therefore the secondary effects obtained with different drugs are expected to be different as well. In this context, TIP constitutes an alternative to the use of conventional drugs, such as the ones mentioned above.

For use as either therapeutical agent or research tool, the compounds should be able to enter the cells and reach their target(s). The compounds according to the invention are efficiently taken up by living cells from the medium and transported into the cell nucleus, as shown in Example 15 herein.

The discovery and commercialization of protein transduction domains frees researchers from transfection troubles

There are many fast and effective methods to introduce transcriptionally active DNA into cells, but options for delivering functional proteins into cells are limited. New research and commercially available products offer another approach: protein transduction.

5 Protein transduction is the process by which peptide or protein motifs cross the cellular plasma membrane. The direct application of functional peptides and proteins to cells has been used to probe signal transduction pathways, block transcription factors, and conduct detailed structure/function analyses of integrin and other receptors' cytoplasmic domains, among other research applications (J. Hawiger, Current Opinion in Chemical Biology, 3:89-94, 1999).

10 One of the main advantages of protein transduction over DNA transfection as a method to express proteins in cells is that, whereas some mammalian cells are 15 notoriously difficult to transfect, all mammalian cells tested to date are receptive to protein transduction. In addition, transduction occurs rapidly, with the protein detected in about 10 minutes rather than after several hours or days. Finally, by varying the amount of protein added to the culture medium, researchers can control the final 20 intracellular protein concentration.

25 Protein transduction is not the only protein-delivery method. Scientists can achieve the same effect using other, more invasive techniques. But the applicability of protein transduction to a wide range of cell types, the speed and ease of translocation across the plasma membrane, and the lack of toxicity of the process, make transduction an 30 attractive option. As a result of the recent explosion in transduction research, a growing range of protein delivery products and kits are coming onto the market.

Other limitations of these methods include the efficiency and cell-to-cell variability. Electroporation, for instance, delivers proteins to only 1-2% of a primary cell population and 10-15% of an established cell line population. Thus, the cells that do not receive the protein can easily mask any effect produced by those cells that do.

Further, not all of the cells that take up protein end up with the same concentration of exogenous protein, leading to variable and difficult-to-interpret results.

The serendipitous observation that some proteins can enter cells when added to the surrounding media, led to the identification of short basic peptide sequences from these proteins that can traverse the plasma membrane and carry the rest of the protein with them (S.R. Schwarze, S.F. Dowdy, "In vivo protein transduction: Intracellular delivery of biologically active proteins, compounds and DNA," Trends in Pharmacological Sciences, 21:45-8, February 2000). The three most widely studied protein transduction domains (PTDs) are derived from the *Drosophila* homeotic transcription factor *Antennapedia* (Antp), the herpes simplex virus (HSV) protein VP22, and the human immunodeficiency virus (HIV)-1 transcriptional activator Tat. Other proteins, such as the *Drosophila* homeotic factors *Fushi-tarazu* and *Engrailed*, contain peptides with similar properties (K. Han et al., Molecules and Cells, 10:728-32, 2000).

#### Protein Transduction Domains

PTDs are generally short peptides, about 10-16 residues in length. Structurally dissimilar, their only common feature appears to be the presence of numerous 20 positively charged lysine and arginine residues (S.R. Schwarze, S.F. Dowdy, Trends in Pharmacological Sciences, 21:45-8, February 2000). PTD-mediated transduction evidently occurs by a mechanism other than classical endocytosis: Cells can take up these peptides at 4°C and in the presence of cellular transport process inhibitors (S.R. Schwarze, S.F. Dowdy, Trends in Pharmacological Sciences, 21:45-8, February 2000).

PTD-mediated transduction seems to be independent of protein size. PTDs have delivered covalently attached proteins in excess of 700 kDa to cells. They have also transduced liposomes over 200 nm in diameter-about the size of a mitochondrion-directly across the cell membrane by anchoring PTDs to the liposome surface.<sup>6</sup> If 30 desired, the covalent attachments that link the PTD to its cargo can be made reversible,

for example, by using disulfide or ester linkages that are reduced or cleaved once inside the cell.

PTD-mediated Tat transduction occurs through a poorly defined mechanism that is independent of receptors, transporters, and endocytosis. When added exogenously to cells, the 101-residue-long Tat protein crosses the plasma membrane, localizes to the nucleus, and initiates the replication of viral DNA. Tat's PTD consists of an arginine-rich stretch of nine amino acids (RKKRRQRRR) embedded within the protein.

When synthesized as a recombinant fusion protein or covalently cross-linked to other peptides or proteins, Tat is capable—albeit with poor efficiency—of delivering biologically active cargo to a cell. However, if the cargo is instead covalently linked at the N- or C-terminus to a truncated Tat PTD, the transduction efficiency improves dramatically. Transduction then occurs via a rapid process that targets cells in a concentration-dependent fashion at both 37°C and 4°C.

Tat-coupled proteins have been transduced into cells and tissues *in vivo*, as well as in culture. It has been demonstrated that intraperitoneal injection by the Tat peptide into a live mouse can deliver functional protein—in this case,  $\beta$ -galactosidase (120 kDa)—to all murine tissues tested, including the brain (S.R. Schwarze et al., *Science*, 285:1569-72, 1999). Only proteins that are covalently linked to the PTD can get in and out of cells, thus ensuring that the process is specific—a particularly important consideration for *in vivo* applications of this technology.

Structural analyses of naturally occurring PTDs have allowed researchers to design an estimated 50 to 70 synthetic, model PTDs, some as short as six amino acids (J.S. Wadia, S.F. Dowdy, *Current Opinion in Biotechnology*, 13[1]:52-6, Feb. 1, 2002). Dowdy et al has developed a Tat derivative (YARAAARQARA) that is 33-times more efficient than the Tat peptide itself, and which can deliver small molecules *in vivo* (A. Ho et al., *Cancer Research*, 61:474-7, Jan. 15, 2001).

Natural and synthetic PTDs must, in most cases, be covalently coupled to their cargo, which is a potential limitation of the technology. However, the Chariot-reagent from Carlsbad, Calif.-based Active Motif Inc (M.A. Wingard, *The Scientist*, 14[22]:19, Nov. 13, 2000), is based on a different protein delivery system, which uses a short synthetic signaling peptide, called Pep-1, but which does not require covalent coupling to the protein or peptide cargo.

Pep-1 is a 21-residue-long carrier consisting of three domains: a hydrophobic, tryptophan-rich motif that targets the cell membrane and forms hydrophobic interactions with proteins; a hydrophilic, lysine-rich domain derived from the simian virus 40 large T antigen nuclear localization sequence, which improves intracellular delivery and solubility of the peptide vector; and a spacer sequence (M.C. Morris et al., *Nature Biotechnology*, 19:1173-6, December 2001). When Pep-1 is mixed with peptides or proteins for 30 minutes in buffer, the molecules rapidly associate through noncovalent hydrophobic interactions and form stable complexes, each having an excess of carrier peptide over the cargo. For example, one molecule of green fluorescent protein (GFP; 30 kDa) interacts with 12-14 molecules of Pep-1 (M.C. Morris et al., *Nature Biotechnology*, 19:1173-6, December 2001). Scientists add these complexes to cells in culture, and two hours later, they can detect the protein *in situ*.

#### **Therapeutic Applications**

In addition to its use as a research tool, the compounds according to the present invention may also have a potential application to clinical medicine. While research in this field is still in its infancy, it is possible that protein delivery will have some application to current gene therapy protocols, in cases where the direct delivery of the gene product itself may be more beneficial than the delivery of the gene.

In addition, protein transduction technology has potential applications for vaccine development. For instance, Phagen has licensed its VP22 technology to Palo Alto, Calif.-based Genencor International Inc. for the development of therapeutic vaccines against hepatitis B, hepatitis C, and human papilloma virus.

Finally, the technology has potential drug delivery applications. Jonathan B. Rothbard and colleagues at Cellgate Inc. of Sunnyvale, Calif., have developed a range of synthetic peptide derivatives, or peptoids, that can be covalently linked to small drugs for their delivery and uptake by cells at different sites in the body (P.A. Wender et al., PNAS, 97:13003-8, 2000).

According to the above and with guidance from the present description, the compounds according to the invention may be used as a vehicle for protein transduction.

The following examples show that the assay in which test compounds are brought in contact with preformed complexes between actin and Hrp65 is useful for identifying such compounds which will inhibit said interaction. They further show that compounds which inhibit said interaction also inhibit transcription, by the demonstration of inhibition of the formation of transcription-active BR-puffs and by the inhibition of BrUTP incorporation into nascent RNA.

To further analyze the actin-Hrp65-2 binding specificity, two peptides bearing double mutations likely to affect actin binding (65-2mut-1 (hereinafter also termed TIP-1, sequence shown in SEQ ID NO 2) and 65-2mut-2 (hereinafter also termed TIP-2, sequence shown in SEQ ID NO 3) were designed (Figure 1D). The sequences of TIP, TIP-1 and TIP-2 are shown below, and also in the sequence listing. The sequences are shown in N- to C-terminal direction and differences to TIP in bold.

TIP	<b>CPYVNQRPQKARYRNG</b>
TIP-1	<b>CPYVNQAPQAARYRNG</b>
TIP-2	<b>CPYVNQRPQKAAYANG</b>

118B CYNDRGNREDRYNNFG

In the experiment presented in Figure 1, these two mutated peptides TIP-1 and TIP-2, the wild type TIP, and two other synthetic peptides of unrelated sequence (118B and 2D13) were covalently coupled to Sulpholink beads (Pierce) via terminal cysteines and used in reconstitution experiments. Following pre-equilibration in G-buffer, the peptide-coupled beads were incubated with saturating amounts of  $^{35}$ S-actin. The bound actin was resolved by SDS-PAGE, detected by autoradiography, and quantified by phosphoimaging (Figure 1F). Virtually no actin was recovered in the bound fraction when actin was incubated with Sulpholink beads alone and only 15% and 11% was recovered with the unrelated peptides 118D and 2D13, respectively. Instead, 75% of the input actin was bound to the TIP-beads and only 10% to the TIP-1 beads. These results show that actin selectively binds to the C-terminal part of Hrp65-2 protein.

An actin/Hrp65-assay performed *in vivo* is also described. It is useful to perform *in vivo* cross-linking experiments to establish whether a compound can disturb the interaction between actin and Hrp65 *in vivo*.

*C. tentans* tissue culture cells were treated *in vivo* with the cross-linker DSP, and nuclear protein extracts were assayed by immuno-precipitation using the monoclonal antibody 4E9 against Hrp65. The bound fractions were studied by Western blotting using polyclonal antibodies against either Hrp65 or actin. As shown in Figure 2A, actin was co-immunoprecipitated from DSP-treated extracts even in the presence of urea (lane 4), which indicated direct actin-Hrp65 cross-linking, and hence direct actin-Hrp65 interaction *in vivo*. A compound which will disturb the interaction between actin and Hrp65 will be readily identified by this method.

TIP was also injected into the nucleus of salivary gland cells of *C. tentans* and the effects of the injection on the expression of the BR genes were analyzed. The BR

genes display all the typical features of protein-coding genes and their expression can be easily monitored at the cytological level using immunofluorescence microscopy. As shown in Figure 3A (panel a), two giant BR puffs, BR1 and BR2, are observed under normal growth conditions in the nuclei of salivary gland cells immuno-stained with antibodies against an abundant non-shuttling SR protein, Hrp45. Microinjection of the 65-2CTS peptide resulted in a pronounced regression of the BR puffs (panel c), an effect comparable to that of actinomycin D (compare panel a with b and c), a drug known to inhibit transcription and to cause a drastic reduction in the size of the BR puffs. Injection of control peptides does not have any visible effect either on the size of the BR puffs or in the overall distribution of Hrp45 (panel d). These observations show that TIP specifically inhibits transcription.

Next, the inventors combined the peptide injections with BrUTP incorporation under conditions that reveal transcription by RNA polymerase II. Under normal conditions, BrUTP injected into the salivary gland cells is effectively incorporated into the BR puffs as well as into a large number of transcriptionally active genes in the chromosomes (Figure 3B, panel e). The BrUTP incorporation was abolished by actinomycin D (panel f). Co-injection of TIP and BrUTP into the same cell drastically reduced BrUTP incorporation (Figure 3, panel g). The effect of TIP was highly specific as judged by the normal levels of BrUTP incorporation observed when control peptides were injected under the same experimental conditions (panel h). Altogether, the results show that the overall RNA synthesis by RNA polymerase II is drastically down-regulated by TIP in a highly specific manner. Furthermore, the effect of TIP is global, not restricted to a small subset of genes.

25

### Examples

In the following examples, chemicals, equipment and reagents are either commercially available or easily prepared by a person skilled in the art. Furthermore, the methods and techniques used in the examples are standard techniques and recipes and protocols can be found in any compilation of methods in cell and molecular biology, see e.g.

30

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Sambrook et al or Current Protocols in Molecular Biology. It should be noted that the experiments are not intended to limit the scope of the invention, they are only intended to illustrate the invention.

5 **Figure legends**

**Figure 1.** Actin Binds Directly to the C-Terminus of Hrp65-2. **a**, Nuclear actin-associated proteins studied by DNase I affinity chromatography and Western blotting. Nuclear extracts were prepared from *C. tentans* tissue culture cells and either fractionated by SDS-PAGE (N), or mixed with DNase I-Sepharose beads. The proteins in the DNase I-bound fraction (B) were eluted and separated by SDS-PAGE. Gels were silver stained (lanes 1 and 2) or analyzed by Western blotting using mAbs against three *C. tentans* proteins: Hrp23 (lanes 3 and 4), Hrp36 (lanes 5 and 6), and Hrp65 (lanes 7 and 8). The actin band previously identified by mass spectrometry is marked with a dot next to the silver stained gel. **b**, Schematic structure of the Hrp65 isoforms containing an N-terminal T7 tag. **c**, Reconstitution experiments using purified  $^{35}\text{S}$ -Hrp65 isoforms immobilized on T7-beads and saturating amounts of  $^{35}\text{S}$ -labelled G-actin. Bound proteins were separated by SDS-PAGE and visualized by autoradiography. **d**, Specific binding of actin to Hrp65-2.  $^{35}\text{S}$ -labelled Hrp65-1 (lanes 1-4) and 65-2 (lanes 5-8) were individually immobilized on T7-beads and incubated with  $^{35}\text{S}$ -labelled purified G-actin in the presence of either non-conjugated KLH (lanes 3 and 7, respectively) or 65-2CTS conjugated KLH (lanes 2 and 6, respectively) to compete binding of actin to the Hrp65 isoforms. The bound proteins were separated by SDS-PAGE and analyzed by autoradiography. **e**, Primary sequences of the peptides used in binding studies. **f**, Specificity of the actin-Hrp65-2 association assayed by affinity chromatography. The wild type 65-2CTS peptide, two mutated versions (65-2mut-1 and 65mut-2) and two unrelated peptides (118B and 2D13) were coupled to Sulpholink beads via their terminal cysteines, and incubated with purified  $^{35}\text{S}$ -labelled G-actin. Bound actin was fractionated by SDS-PAGE and visualized by autoradiography. The bound actin was quantified by phosphoimaging and the results are given under each lane as percentages of input actin.

Figure 2. Actin Binds to Hrp65 *In Vivo*. a, Co-immunoprecipitation after *in vivo* cross-linking. Cultured *C. tentans* cells were incubated in the absence (lanes 1 to 4) or the presence of peptides 65-2CTS (lanes 5 and 6) and 118B (lanes 7 and 8), and then treated with the cell permeable cross-linking reagent DSP. Nuclear extracts were prepared, and each extract was split into two equal portions, one being treated with urea. Nuclear extracts were also prepared from DSP-untreated cells (lines 1 and 2). All samples were subjected to immunoprecipitation with mAb 4E9 against Hrp65, and bound proteins were analyzed by SDS-PAGE and Western blotting using rabbit polyclonal antibodies against Hrp65 and actin. b, Peptides 65-2CTS and 118B are spontaneously internalized into *C. tentans* tissue culture cells. Peptides were labeled with fluorescein maleimide and added to the cell medium at a final concentration of 15  $\mu$ M. The internalization of the peptides was analyzed by confocal microscopy. Fluorescein-labelled 65-2CTS was detected almost exclusively in the nucleus (cf. panels a, e and i) whereas fluorescein-labelled 118B is found in both the nucleus and the cytoplasm (cf panels c, g and k). In control experiments, the fluorescein labeled 65-2CTS (cf. panels b, f and j) and 118B (cf. panels d, h and l) were digested with trypsin before incubation with the cells.

Figure 3. Disruption of the Actin-Hrp65-2 Complex Down-Regulates mRNA Transcription. a, Salivary glands were isolated from *C. tentans* fourth instar larvae, immunostained with mAb 2E4 against Hrp45, and visualized by confocal microscopy. a-d, show individual salivary gland cells untreated (a), treated with the transcription inhibitor actinomycin D at 4  $\mu$ g/ml for 90 min before immunostaining (b), injected with peptide 65-2CTS (TIP) at 30-60 mg/ml before immunostaining (c), or injected with control peptides, either 118B or 65-2mut1, at 30-60 mg/ml before immunostaining (d). After peptide injections, the glands were incubated in hemolymph at 18°C for 90 min before fixation and immunostaining. For each treatment, three examples are presented: one showing a full nucleus and two additional ones showing only the BR puffs. The bar in a represents 10  $\mu$ m and all photographs are at the same

magnification. e-h, Detection of nascent transcripts by incorporation of BrUTP.

BrUTP was injected into the cytoplasm of salivary gland cells at 100 mg/ml, and the incorporation of BrUTP into nascent RNA was visualized by immunofluorescence after 20 min incubation in hemolymph at 18°C, using a mouse anti-BrUTP antibody and a FITC-coupled secondary antibody. In f, the glands were incubated with hemolymph containing actinomycin D at 4 µg/ml for 60 min before BrUTP injection and immunostaining. In g, h, either peptide 65-2CTS (TIP) or control peptides (65-2mut-1 or 118B) were injected into the nucleus of the salivary gland cells, the glands were incubated in hemolymph for 60 min at 18°C, and BrUTP was administered by cytoplasmic injection. The glands were then incubated for additional 20 min prior to fixation and immunostaining as above.

**Figure 4. Specificity of the Anti-Hrp65-2 Antibody.** a, *C. tentans* nuclear proteins probed with either antibody 282-296 against all the Hrp65 isoforms (lane 1), or the peptide-specific antibody raised against the C-terminal sequence of Hrp65-2 (lane 2). The mobility of molecular mass standards is given in kDa. b, Polytene chromosomes were manually isolated as described by Björkroth et al. and immunostained with the Hrp65-2 specific antibody followed by a gold-conjugated secondary antibody and silver enhancement. In some cases, the isolated chromosomes were treated with 100 µg/ml RNase A for 60 min at room temperature prior to post-fixation and immunostaining. Phase contrast photographs of the immunostained chromosomes are provided under the bright field images. The bar represents 10 µm.

**Figure 5. Hrp65-2 is Associated with the Nascent BR pre-mRNP Particles. (A)**

Immuno-electron microscopic localization of Hrp65-2 in the BR transcription unit. Polytene chromosomes were isolated as in Figure 4, incubated with the anti-Hrp65-2 antibody at 0.5 mg/ml, and detected with a secondary antibody conjugated to 12-nm colloidal gold markers. The immunolabeled chromosomes were fixed, dehydrated, embedded in Agar resin and sectioned as previously described. a, Distribution of gold markers in the proximal (p), middle (m) and distal (d) portions of the BR transcription

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unit. b, Schematic representation of the BR transcription unit showing the progressive growth of the pre-mRNPs along the gene and their release into the nucleoplasm upon transcription termination. c-h, Examples of immunolabeling in nascent BR pre-mRNP particles. Schematic interpretations of the images are provided under each micrograph.

5 The bar represents 100 nm.

#### Figure 6

10 The figure demonstrates the effect of TIP on the incorporation of  $^{32}\text{P}$ -ATP isotope into newly synthesized RNA. It is clear that there is a dramatic reduction of the total RNA produced when the peptide is added (squares on the chart vs triangles for the control).

#### Definitions

15 It is to be understood that the terminology used herein is for the purpose of describing particular embodiments and aspects of the invention only, and is not intended to limit the scope of the invention.

20 Throughout this specification and the claims, the words "comprises" and "comprising" are used in a non-exclusive sense.

25 It should be noted that, as used herein and in the appended claims, the singular forms "a", "an" and "the" include plural reference unless the context clearly dictates otherwise.

30 Unless defined otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of ordinary skill in the art to which this invention belongs.

35 Amino acids are denoted by the conventional one-letter abbreviations. Polypeptides are written in N-terminal to C-terminal direction.

As defined herein, the terms "similarity" or "similar substitutions" mean that chemically similar amino acids replace each other. For example, the basic residues Lys and Arg are considered chemically similar and often replace each other, as do the acidic residues Asp and Glu, the hydroxyl residues Ser and Thr, the aromatic residues Tyr, Phe and Trp, and the non-polar residues Ala, Val, Ile, Leu and Met. Similarity is measured by dividing the number of similar residues by the total number of residues and multiplying the product by 100 to achieve a percentage.

5 By "identity" is meant a property of sequences that measures their similarity or relationship. Identity is measured by dividing the number of identical residues by the total number of residues and multiplying the product by 100 to achieve a percentage. Thus, two copies of exactly the same sequence have 100% identity, but sequences that are less highly conserved and have deletions, additions, or replacements may have a 10 lower degree of identity. Those skilled in the art will recognize that several computer 15 programs, such as those that employ algorithms such as BLAST (Basic Local Alignment Search Tool, Altschul et al. (1993) J. Mol. Biol. 215:403-410) are available for determining sequence identity.

20 As defined herein, the term "Hrp65-like activity" means the ability of a compound to interact with actin, in the same way as Hrp65 does. It is well within the capacity of the skilled person to determine, with reference to the present description, whether a compound is capable of interacting with actin or not. A compound with Hrp65-like activity is normally a protein, similar or identical to the molecule disclosed in SEQ ID 25 NO 4.

As defined herein, the terms Hrp65 and Hrp65-2 are used interchangeably, and are used to refer to the amino acid sequence shown in SEQ ID NO 4.

30 As defined herein, the term "TIP" or "transcription inhibiting peptide" relates to a compound chosen from the group which comprises; the polypeptide according to SEQ

15 ID NO 1 or analogues thereof, oligomers thereof, fragments thereof, or oligomers of fragments.

20 As defined herein, the term "analogue", in the context of the TIP polypeptide, is meant a polypeptide having TIP-like activity, in which polypeptide one or more amino acids are replaced by a different, natural or artificial, amino acid. Also included are variants of TIP in which deletions, substitutions, additions or repeats of one or more amino acids have been introduced. Furthermore, fragments of the peptide, or oligomers of these fragments are included.

25 10 As defined herein, the terms "TIP", "65-2CTS" or "transcription inhibiting peptide" may be used interchangeably and are meant to refer to the polypeptide shown in SEQ ID NO 1.

30 15 As defined herein, the terms "TIP-activity", "TIP-like activity", or "transcription inhibiting activity" relate to the activity of TIP which inhibits transcription and consequently cell proliferation. The terms relate to the effect TIP has on transcription. It is well within the capacity of the skilled person to determine, with the help of the description given herein, whether or not a compound has any transcription inhibiting activity. A compound will be considered to be an inhibitor of transcription if it blocks transcription at least by 30%, preferably at least by 40%, more preferably at least by 50% or most preferably at least by 60%, compared to control experiments. Another definition of an inhibitor may be one which, at a concentration of 10  $\mu$ M, or preferably 1  $\mu$ M, will disrupt 50% of the complexes between actin and Hrp65. Yet another definition of an inhibitor will be one which at a concentration of 10  $\mu$ M, or preferably 1  $\mu$ M, will decrease the incorporation of BrUTP into RNA by 50%.

35 20 As defined herein, the term Hrp65 refers to the polypeptide according to SEQ ID NO 4, and to functional equivalents thereof. Being a functional equivalent of Hrp65 implies that a molecule has essentially the same actin-interacting property as Hrp65. It is well within the capacity of the skilled person, with reference to the description

disclosed herein, to establish whether a molecule interacts with actin or not. Consequently, variants are included in the scope of the invention. Such variants include polypeptides according to SEQ ID NO 4, in which additions, deletions, or substitutions have been made, while still retaining the Hrp65-like activity.

5 The treatments may include various "unit doses." Unit dose is defined as containing a predetermined-quantity of the therapeutic composition. The quantity to be administered, and the particular route and formulation, are within the skill of those in the clinical arts. A unit dose need not be administered as a single injection but may 10 comprise continuous infusion over a set period of time. Unit dose of the present invention may conveniently be described in terms of mg/kg body weight.

15 **Example 1**

**Antibodies**

10 TIP, (SEQ ID NO 1) corresponding to the C-terminal sequence of Hrp65-2, amino acids 503-517, plus an N-terminal cystein, was conjugated to keyhole limpet hemocyanin (KLH) and used to immunize rabbits according to standard procedures. The presence of anti-Hrp65-2 antibodies in the sera was analyzed by Western blotting 20 against recombinant Hrp65-2 and against nuclear extracts prepared from *C.tentans* cultured cells. The anti-Hrp65-2 antibody was purified by peptide-affinity chromatography using the UltraLink system (Pierce). The activity of the affinity purified antibody was confirmed by ELISA assay. For microinjection assays, the purified antibody was further concentrated using a Nanosep centrifugal device (Pall 25 Gelman Sciences).

**Example 2**

**Peptide Synthesis**

30 All peptides (65-2 CTS, 65-2 CTS mut-1, 65-2 CTS mut-2, 118B and 2D13) were chemically synthesized by solid phase method as described (Atherton & Sheppard,

1989) and purified by reverse phase HPLC to more than 80% homogeneity. Peptides may also be purchased from Innovagen, Lund, Sweden. Mass spectral analysis was performed to confirm their expected molecular weights.

5 **Example 3**

**Plasmids construction**

A Bgl II-Sal I restriction fragment containing the full ORF of *hrp65-2* was excised from pEGFP-C3-*hrp65-2* (Miralles and Visa, 2001) and cloned into the Bam HI – Sal I sites of pET21b (Novagen) to generate pET21b-*hrp65-2*.

10

**Example 4**

**Fluorescent labeling of peptides and proteins**

Aliquots of peptides or proteins were reacted with fluorescein-5-maleimide (obtainable from Roche) via a cysteine amino acid residue placed at the N-terminal end of the TIP and 118B peptides, or at cysteine residues on either Hrp65 or actin. 1-3 mg of peptides TIP and/or 118B or proteins dissolved in PBS were incubated with 2 eq of fluorescein-5-maleimide dissolved in dimethylformamide (DMF) per SH group of the peptide. The labeling reaction was carried out for 2 hours at room temperature with continuous agitation and in the dark. Fluorescent peptides or proteins were purified on a G-50 spin column and stored at -20°C in the dark until further use.

20

**Example 5**

**Preparation and labeling of proteins for protein-protein interaction assay**

<sup>35</sup>S-labelled native actin from non-muscle cells was prepared by DNase I affinity chromatography essentially as previously described (Zechel, K., (1980)).

25

The plasmid containing the full-length ORF of *hrp65-2*, (pET21b-*hrp65-2*, encoding the amino acid sequence according to SEQ ID NO 4) was used as template for TNT-coupled *in vitro* transcription/translation essentially following the manufacturer's instruction manual (TNT Coupled Reticulocyte Lysate Systems, Promega). The

30

proteins were labeled by incorporation of  $^{35}\text{S}$ -methionine, electrophoresed in 10% SDS/polyacrylamide gels and detected by autoradiography.

#### Example 6

##### Culturing Conditions and Preparation of Extracts from *C. tentans* Tissue Culture Cells

*C. tentans* was reared as described by Case and Daneholt (1978), and salivary glands were isolated from fourth instar larvae. *C. tentans* tissue culture cells were grown in suspension at 24°C as devised by Wyss (1982).

*C. tentans* extracts were prepared as described by Wurtz et al. (1996). Tissue culture cells were washed with PBS and re-suspended in PBS containing 0.2% NP40 and 0.1 mM PMSF. The cells were homogenized and spun at 2000 g for 5 minutes at 4°C. The supernatant constituted the cytosolic extract. The pellet was washed twice in PBS and resuspended in PBS containing 0.1 mg/ml tRNA, sonicated, and clarified. The supernatant was retained as the nuclear extract.

#### Example 7

##### Microinjection experiments

Microinjections were performed on isolated salivary glands. The peptides were injected at a concentration of 30mg/ml or 60mg/ml in PBS. After injection, the glands were incubated at 18°C for 90 min in hemolymph and fixed. Immunofluorescent detection of hrp45 was performed as described herein.

#### Example 8

##### SDS-PAGE and Western Blot Analysis

Proteins were fractionated on 10% polyacrylamide gels, containing 0.1% SDS (SDS-PAGE), transferred electrophoretically to PVDF membranes (Immobilon-P; Millipore) in a semi-dry transfer cell (Trans-Blot; Biorad) and visualized with 0.2% (w/v) Ponceau-S (Biorad) in 10% (v/v) acetic acid. After blocking with 10% (w/v) non-fat dry milk in PBS-0.1% Tween-20, the blots were incubated with monoclonal antisera

against hrp36 and hrp65 (1:200 dilution) in 5% (w/v) non-fat dry milk in PBS-0.1% Tween-20. Both hrp36 and hrp65 were detected using a goat anti-mouse IgG (DAKO) coupled to horse-radish peroxidase and immunodetection was performed using the enhanced chemiluminescent method as described in the manufacturer's instruction manual (ECL-Plus, Amersham).

#### 5 Example 9

##### Affinity chromatography

Different "pull-down" assays were used to establish *in vitro* interactions between the 10 hrp65 isoforms, namely hrp65-1, 65-2 and 65-3 and actin. Since both full-length proteins tagged with specific epitopes and short synthetic peptides with terminal Cys residues were used in *in vitro* binding studies with actin, the experimental set-up used is described below in terms of the type of bead/resin involved.

15 DNase I (Sigma) was coupled to CNBr-activated Sepharose (Pharmacia) according to the manufacturers instructions. Typically, 300 µl DNase I beads pre-equilibrated in 1xPBS were incubated in a single step procedure nuclear and cytosolic extracts, prepared from 500 ml of *C. tentans* tissue culture cells. Following incubation for 30-40 minutes at 4°C, the beads were repeatedly washed to remove non-specifically bound 20 material as previously described. The beads were resuspended in Laemmli buffer, heat denatured to elute the bound proteins which were then resolved by 10% SDS-PAGE. The proteins were detected by silver staining. The results are shown in figure 1A.

##### T7 tag antibody agarose co-immunoprecipitations

25 T7-tagged  $^{35}$ S-labelled hrp65-1, hrp65-2 were expressed by coupled *in vitro* transcription/translation and immobilised onto T7-tag antibody agarose following the instruction manual. Hrp65 agarose beads were then incubated with saturating amounts of DNaseI affinity-purified  $^{35}$ S-labelled native actin for 30-40 minutes at 4°C with continuous mixing. In parallel, competition experiments were also performed using 30 Hrp65 beads. Both Hrp65-1 and Hrp65-2 beads were pre-saturated with TIP-peptide conjugated with keyhole limpet hemocyanin (KLH), as well as KLH alone for 20

5 minutes at 4°C with continuous mixing and subsequently incubated with saturating amounts of <sup>35</sup>S-labelled actin as mentioned above. Beads were then spun down and washed with phosphate buffer saline containing 4% glycerol, 1% NP40, 0.1% deoxycholate, 1 mM DTT as already described. Bound proteins were analyzed as described above and detected by autoradiography of the fixed and dried gel. Figure X

#### *Peptide binding assays*

10 Various peptides containing specific and mutant sequences from hrp65-2 (respectively termed 65-2 CTS (TIP), 65-2mut-1(TIP-1), 65-2mut-2(TIP-2) as well as unrelated ones (termed 118B and 2D13) were covalently bound to Sulpholink resin (Pierce) via a C-terminal or N-terminal Cys residue. Typically about 3.0 mg of lyophilized peptide were dissolved in 50 mM Tris, 5 mM EDTA, pH 8.5 and incubated with 1 ml of Sulpholink resin (50% slurry) pre-equilibrated in 50 mM Tris, 5 mM EDTA, pH 8.5 according to the manufacturer's instructions. The coupling efficiency was higher than 90% as detected by UV spectrophotometry. After pre-equilibration in G-buffer (5 mM Tris pH 7.6, 0.5 mM ATP, 0.1 mM CaCl<sub>2</sub> and 0.5 mM DTT), peptide beads were individually incubated with saturating amounts of <sup>35</sup>S-labelled native actin purified by DNase I affinity chromatography for 45 minutes at 4°C with continuous mixing. The beads were then washed with phosphate buffer saline containing 4% glycerol, 1% NP40, 0.05% deoxycholate, in non-reducing conditions. Both bound and unbound proteins were then analysed as already described and the proteins detected by autoradiography (Figure 1F). The results identify TIP as an actin-binding compound.

#### **Example 10**

##### **Assay for identifying compounds capable of inhibiting the interaction between Hrp65 and actin.**

25 Such an assay may comprise the following steps; (a) conjugating Hrp65 to a solid support, such as a bead, surface, or in a well in a multiwell plate, (b) washing away non-conjugated Hrp65, (c) blocking non-conjugated sites on the solid support by adding another protein (e.g. bovine serum albumin, or a blocking mixture such as milk powder), (d) adding labeled (e.g. radioactively, by e.g. <sup>35</sup>S, or fluorescently, by e.g.

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FITC) actin either in the presence or absence of a test compound, (e) washing away  
5 unbound actin, (f) measuring either the amount of actin still bound (e.g. by measuring  
fluorescence) or the amount of unbound actin (e.g. by scintillation counting), (g)  
comparing the amount of bound/unbound actin in the presence of test compound with  
the amount of bound/unbound actin in the absence of test compound. A compound  
which prevents more than half of the actin molecules to bind to Hrp65 at a  
concentration lower than 100 $\mu$ M, or preferably 10 $\mu$ M, or more preferably 1 $\mu$ M, is  
10 considered to be an inhibitor of the actin/Hrp65 interaction and may therefore be  
useful in treating antiproliferative diseases. FITC is fluorescein isothiocyanate, and is  
commercially available from Molecular Probes, or from Sigma. It is also possible to  
15 use an analog of FITC, fluorescein-5-maleimide, which is commercially available  
from Pierce.

#### Example 11

##### Isolation and Immunostaining of Polytene Chromosomes

15 Salivary glands were isolated from fourth instar *C. tentans* in ice-cold TKM buffer  
(100 mM KCl, 1 mM MgCl<sub>2</sub> and 10 mM triethanolamine-HCl, pH 7.0). All isolation  
steps were performed at a temperature close to 0°C if not indicated otherwise. The  
20 glands were incubated for 60 sec in TKM buffer, containing 2% Nonidet P40 (NP-40),  
and transferred twice into fresh 0.025% NP-40 in TKM. The polytene chromosomes  
were released from the glands by a pipetting procedure described by Björkroth et al.  
(1988). Individual chromosomes were collected and transferred to a microscopic slide.  
25 After attaching to the surface they were post-fixed in 4% paraformaldehyde in TKM  
for 30 min at room temperature and washed three times with fresh TKM. Prior to  
fixation, some of the chromosomes were treated with RNase A (100  $\mu$ g/ml) for 60 min  
at room with subsequent postfixation and washes as described above. For  
immunocytochemical analysis the isolated chromosomes were blocked with 2% bovine  
30 serum albumin (BSA) in TKM for 30 min at room temperature in a humid chamber.  
The primary antibody, anti-hrp65-2, was applied diluted 1:100 in TKM containing  
0.5% BSA, and incubated for 60 min. As negative control a non-related antibody  
against synaptonemal complex protein 3 (SCP3) was used. The slides were washed

three times for 5 min each with 0.1% Tween-20 in TKM, with subsequent incubation for another hour with a 6 nm gold-conjugated secondary antibody (Jackson ImmunoResearch Laboratories) diluted 1:50 in 0.5% BSA in TKM buffer. The specimens were washed three times for 5 min each in TKM and distilled H<sub>2</sub>O. For visualization of the gold particles an immunogold silver enhancement solution (IntenSE™ M, Amersham) was added onto the chromosomes for 5-10 min at room temperature. The reaction was terminated with water and the specimens were mounted in 30% glycerol and examined and photographed under a light microscope (Carl Zeiss). The results of this experiment are shown in Figure 4b.

10

### **Example 12**

#### **Immunoelectron Microscopy**

15

Polytene chromosomes were isolated and immunostained as described above, but with some differences. The chromosomes were transferred to a siliconized slide and the colloidal gold on the secondary antibody had a diameter of 12 nm. After labeling with the primary and secondary antibody the chromosomes were fixed with 2% glutaraldehyde in TKM for 60 min. They were washed with TKM for three times, 5 min each, dehydrated in three steps with ethanol (90, 95, 100%) and with subsequent embedding in an Agar 100 resin. The specimens were sectioned (60-75 nm) with a Leica Ultracut ultramicrotome (Leica, Wien, Austria) and the sections were stained with saturated uranyl acetate and subsequently with lead citrate. The individual gold particles along the BR genes were studied in a Philips CM120 electron microscope at 60 kV. The results of this experiment are shown in Figure 5.

20

### **Example 13**

#### ***In Vivo* protein-protein cross-linking**

25

Proteins in *C. tentans* tissue culture cells were cross-linked for 20 min at room temperature with 0.5 mM dithiobis-succinimidylpropionate (DSP; Sigma/Aldrich) in the standard cultivation medium. Nuclear and cytosolic extracts were prepared as described above and where appropriate incubated with 8 M urea. The extracts were diluted 10x with PBS, containing 0.2% NP-40 and 1mM PMSF, and immediately

30

incubated with a monoclonal antibody against hrp65 termed 4E9 to co-immunoprecipitate hrp65 and actin. This experiment is shown in Figure 2A.

#### Example 14

##### Fluorescence microscopy

###### *Immunofluorescence of C. tentans salivary gland cells*

*C. tentans* salivary glands were isolated from the fourth instar larvae as described (Lezzi et al., 1981). For indirect immunofluorescence microscopy, isolated salivary glands were fixed with 4% formaldehyde in PBS for 10 min on ice and further incubated with 4% formaldehyde and 5% Triton X-100 for 20 min on ice. Following fixation, the glands were further permeabilised for 10 min at RT with 10% Triton X-100. Samples were then washed three times with PBS and respectively incubated overnight at 4°C with antibodies against hrp45 (1:100 dilution, in PBS containing 0.5% nonfat dry milk and 0.5% BSA) or hrp65-2 (1:20 dilution). Samples were rinsed three times with PBS and then incubated for 1h at RT with the respective secondary antibodies. A FITC-conjugated anti mouse secondary antibody diluted 1:100 in PBS containing 0.5% nonfat dry milk and 0.5% BSA was used to detect the monoclonal antibody against hrp45; a FITC-conjugated anti rabbit antibody (1:50) was used to detect the polyclonal antibody against hrp65-2; a FITC-conjugated anti goat antibody (1:50) was used to reveal the antibody against the large subunit of RNA polymerase II. DAPI was added at the end of the incubation to stain DNA; the samples were then washed three times in PBS and mounted in Mowiol. Images of salivary gland nuclei were taken with a LSM 510 Laser Scanning Microscope. The thickness of the section is 1  $\mu$ M. Before fixation, dissected glands were incubated 90 min in hemolymph with Actinomycin D at a final concentration of 4  $\mu$ g/ml. Immunofluorescence experiments were performed as described above.

#### Example 15

##### Internalization of peptides into living cells

Approximately  $10^7$  *C. tentans* tissue culture cells in suspension were individually incubated for 15 minutes at room temperature with fluorescein-labeled 65-2 CTS and

118B at a final concentration of 5-10  $\mu$ M. Concomitantly, *C. tentans* cells were also  
incubated with fluorescein-labeled 65-2 CTS and 118B peptides pre-digested with  
trypsin for 1h at 37°C. The samples were subsequently washed and spun down on  
poly-lysine coated glass plates for 3 minutes at 800 rpm using a Cytospin apparatus.  
5 Cells were then fixed with 4% formaldehyde in PBS for 10 minutes at room  
temperature, washed in PBS and permeabilized with 0.1% triton-X 100 for 5 minutes  
at room temperature. The samples were then washed with PBS and blocked with 5%  
milk for 20 minutes at room temperature. Sytox (Molecular Probes) was added to the  
cells at a final concentration of 0.25  $\mu$ M to stain the DNA. The samples were washed  
10 with PBS and mounted in Mowiol. Images were recorded with a LSM 510 Laser  
Scanning Microscope as described above. The result shows that TIP is efficiently  
internalized from the culture medium into the cells, and transported to the cell nucleus.  
This experiment is shown in Figure 2b.

15 **Example 16**

BrUTP incorporation shows inhibition of transcription after addition of TIP.  
For transcription experiments, BrUTP (5-Bromouridine 5'-Triphosphate, Sigma) was  
injected at a concentration of 100 mg/ml, 60 min after peptide injections (either TIP or  
control peptides). Incorporation of BrUTP into nascent RNA was visualized by  
20 immunofluorescence after a 90 minute incubation in hemolymph at 18°C, using a  
mouse anti BrUTP antibody (Roche) 1:10 in phosphate buffered saline (PBS)  
containing 0.5% nonfat dry milk and 0.5% BSA) and revealed by a FITC-coupled goat  
anti-mouse antibody (1:100 in PBS containing 0.5% nonfat dry milk and 0.5% BSA).  
The samples were then washed three times in PBS and mounted in Mowiol  
25 (Calbiochem). Images of salivary gland nuclei were taken with a LSM 510 Laser  
Scanning Microscope. The thickness of the optical section was 1  $\mu$ M. The result is  
shown in figure 3e-h, indicating that TIP reduces incorporation of BrUTP into nascent  
transcripts. The figure shows three examples of each treatment, and all the  
photographs are taken at the same magnification.

**Example 17****The effect of TIP on the morphology of the Balbiani ring genes.**

Salivary glands were isolated from *C. tentans* fourth instar larvae, immunostained with monoclonal antibody 2E4 against Hrp45, and visualized by confocal microscopy. The results are shown in figure 3, panels a-d. Individual salivary gland cells were either untreated, treated with the transcription inhibitor actinomycin D at 4 µg/ml for 90 min before immunostaining, injected with peptide 65-2CTS at 30-60 µg/ml before immunostaining, or injected with control peptides, either 118B or TIP-1, at 30-60 µg/ml before immunostaining. After peptide injections, the glands were incubated in hemolymph at 18°C for 90 min before fixation and immunostaining. For each treatment, three examples are presented: one showing a full nucleus and two additional ones showing only the BR puffs. The bar in panel a represents 10 µm and all photographs are at the same magnification.

**Example 18****The effect of TIP on mouse erythroleukemia cells.**

Typically 500 microliters of Murine Erythroleukemia cells (grown in suspension) were incubated with TIP to a final concentration of 0.1 mM. Following a 30 minute incubation at room temperature, 1 microliter of <sup>32</sup>P-ATP isotope (10mCi/ml) was added to the cells and 50 microliter aliquots were taken every two minutes and placed on ice (to stop ongoing transcription). To measure the amount of incorporated radioactive isotope into newly synthesized RNA, poly(A)+ RNA was prepared from each aliquot by oligo(dT)-cellulose affinity chromatography. For this purpose, cell lysis was performed in lysis buffer containing 10 mM Tris pH 7.5, 1 mM EDTA, 0.5% SDS, 0.15 M LiCl, and followed by incubation at 65° C for 5 minutes. Extracts were then left on ice for 5 minutes and then incubated with 20 microliters of pre-equilibrated oligo(dT)-cellulose for 30 minutes at 4° C under continuous agitation. Beads were subsequently washed with a 10-fold excess of lysis buffer (same as above) and the total poly(A)+ RNA bound to the beads was quantified by standard scintillation methods. As control, the same experiment was performed in parallel, but

without peptide. In both cases, the values obtained in cpm (counts per minute) from isotope measurements were then plotted against time. The results are shown in figure 6.

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**Claims**

1. A method for identifying compounds which inhibit, prevent or otherwise disturb  
the interaction between Hrp65 and actin, comprising the steps of:
  - 5 (a) providing an Hrp65 molecule,
  - (b) adding an actin molecule to said Hrp65 molecule, thereby forming a complex  
between actin and Hrp65,
  - (c) adding a test compound to said complex,
  - 10 (d) determining the effect of said test compound on said complex.
2. A method according to claim 1, in which the test compound is added in step (a) or  
(b), together with the Hrp65 molecule or actin molecule, respectively.
- 15 3. A method for identifying compounds which inhibit the interaction between actin  
and Hrp65, comprising the steps of:
  - (a) conjugating Hrp65 to a solid support, such as a bead, resin, surface, or in a well  
in a multiwell plate,
  - (b) removing any non-conjugated Hrp65,
  - 20 (c) blocking non-conjugated sites on the solid support by adding another protein,  
such as bovine serum albumin or milkpowder,
  - (d) adding labeled actin to the solid support conjugated Hrp65,
  - (e) removing actin which has not bound to conjugated Hrp65,
  - (f) optionally calculating the amount of formed actin/Hrp65 complexes,
  - 25 (g) adding a test compound,
  - (h) collecting and measuring the amount of displaced actin,  
in which method the conjugated molecule may be actin, in which case the labeled  
molecule is Hrp65.

4. A method according to claim 3, in which the amount of labeled actin or Hrp65 remaining bound to the conjugated protein is measured, e.g. by immunochemical methods, after step (h).
5. A compound identified by the method according to claims 1-4.
6. A compound according to claim 5 for medical use.
7. Use of a compound according to claim 5 for the manufacture of a medicament to be used in the treatment of hyperproliferative diseases.
8. A compound comprising the amino acid sequence CPYVNQXPQX (SEQ ID NO 5), in which X may be any amino acid.
9. A compound comprising the amino acid sequence CPYVNQXPQX (SEQ ID NO 5), in which X may be any amino acid, which has an inhibiting effect on the interaction between actin and Hrp65.
10. A compound comprising the amino acid sequence CPYVNQXPQX (SEQ ID NO 5), in which X may be R or K, which has an inhibiting effect on the interaction between actin and Hrp65.
11. A compound comprising the amino acid sequence CPYVNQXPQXAXYXNG (SEQ ID NO 6), in which X may be any amino acid, preferably arginine or lysine or alanine, or analogues or oligomers thereof, fragments thereof, or oligomers thereof, or a polypeptide having at least 80% similarity, preferably at least 90% similarity, more preferably at least 95%, further more preferably at least 96%, even more preferably at least 97%, or most preferably at least 98% similarity to said compound, or more preferably at least 80% identity, preferably at least 90% identity, more preferably at least 95%, further more preferably at least 96%, even more preferably at least 97%, or most preferably at least 98%, identity to said

compound, whereby said compound has an inhibiting effect on the interaction between actin and Hrp65.

12. A compound chosen from the group comprising; a polypeptide according to SEQ ID NO 1 or SEQ ID NO 3, which has an inhibiting effect on the interaction between actin and Hrp65.

13. A compound according to claim 9-12, in which the compound is the polypeptide shown in SEQ ID NO 1, which has an inhibiting effect on the interaction between actin and Hrp65.

14. A compound according to any of claims 9-12, which has an anti-proliferative effect.

15. A compound according to any of claims 9-14, for medical use.

16. Use of a compound according to any of claims 9-14, for the manufacture of a medicament to be used in the treatment of hyperproliferative diseases.

17. Use according to claim 16, wherein the condition to be treated is chosen from the group comprising; melanoma, non-small-cell lung cancer, small-cell lung cancer, lung cancer, hepatocarcinoma, retinoblastoma, astrocytoma, glioblastoma, leukemia, neuroblastoma, cancer in the gum, tongue, head, neck, breast, pancreas, prostate, kidney, bone, testicle, ovary, mesothelia, cervix, gastrointestinal tract, lymphoma, brain, colon, sarcoma, bladder, rheumatoid arthritis, inflammatory bowel disease, osteoarthritis, leiomyomas, adenomas, lipomas, hemangiomas, fibromas, vascular occlusion, retenosis, atherosclerosis, pre-neoplastic lesions, adenomatous hyperplasia, prostatic intraepithelial neoplasia, carcinoma in situ, oral hairy leukoplasia, or psoriasis.

18. A pharmaceutical composition comprising a compound according any of claims 9-14 together with pharmaceutically acceptable excipients and additives.

19. Use of a compound according to any of the claims 9-13 in an assay in which transcription of DNA into RNA is inhibited by said compound.

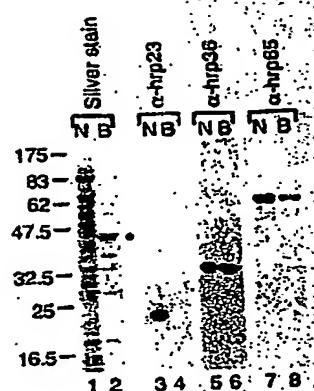
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20. Use of a compound according to any of the claims 9-14, for protein transduction.

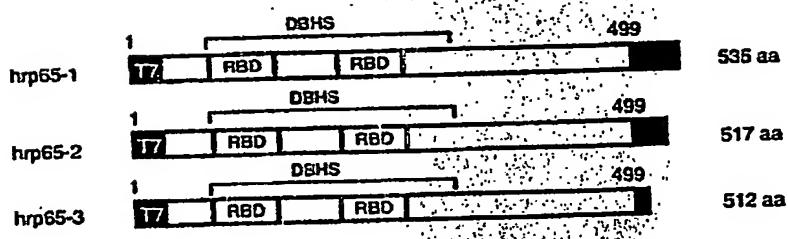
**Abstract**

The inventors have shown that Hrp65 and actin interacts and that this interaction is important for transcription. Consequently, the present invention relates to a novel compound which is useful in the treatment of hyperproliferative diseases. It further relates to the use of the compound for manufacturing a medicament for the use in the treatment of such diseases. Furthermore, the invention relates to a method for identifying other such compounds. Another use of the compounds described herein is as a tool for studying processes in cells. Yet another use of the compounds is for protein transduction.

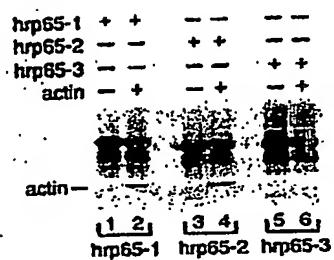
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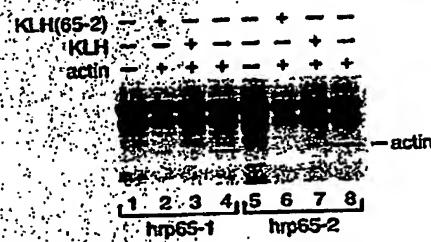
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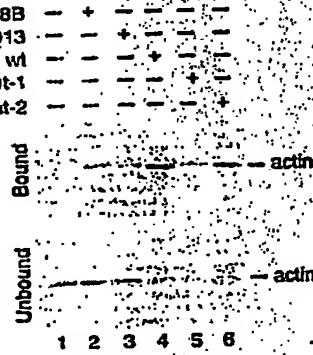
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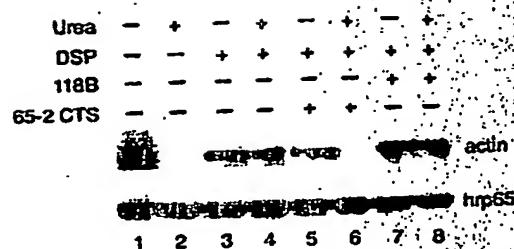
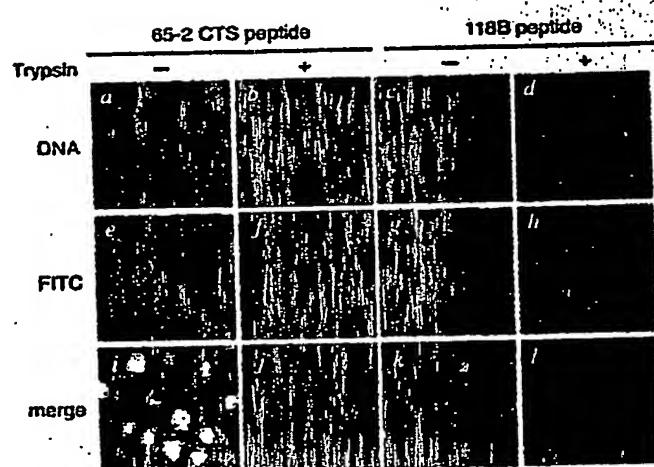


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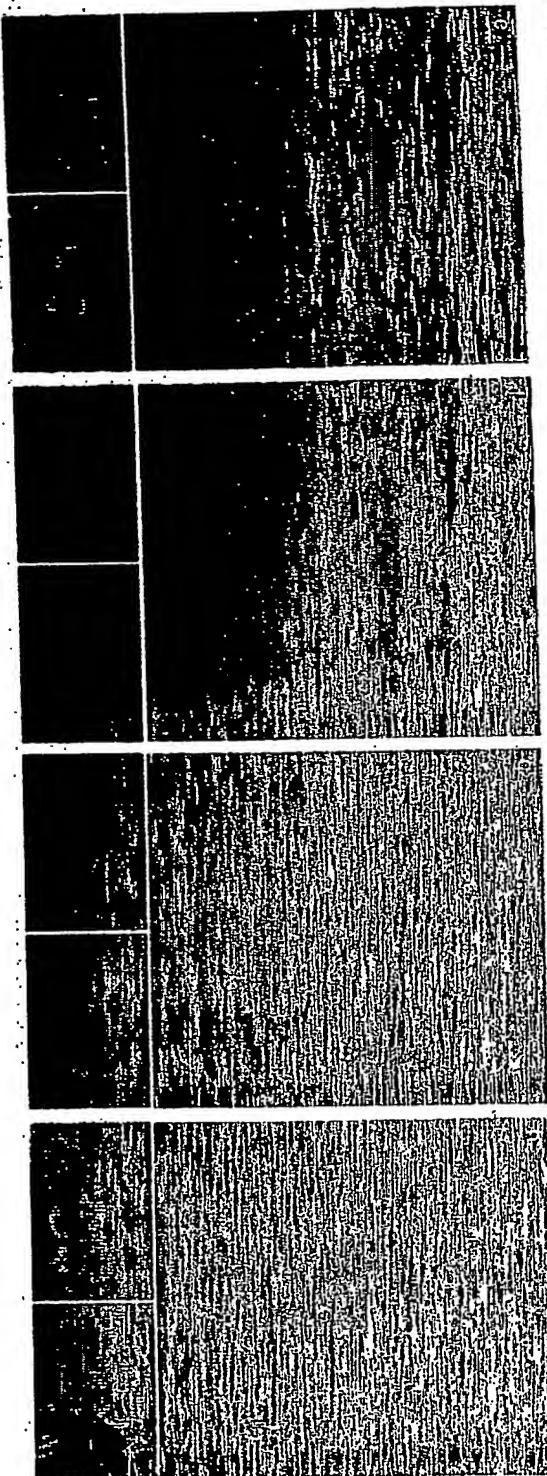
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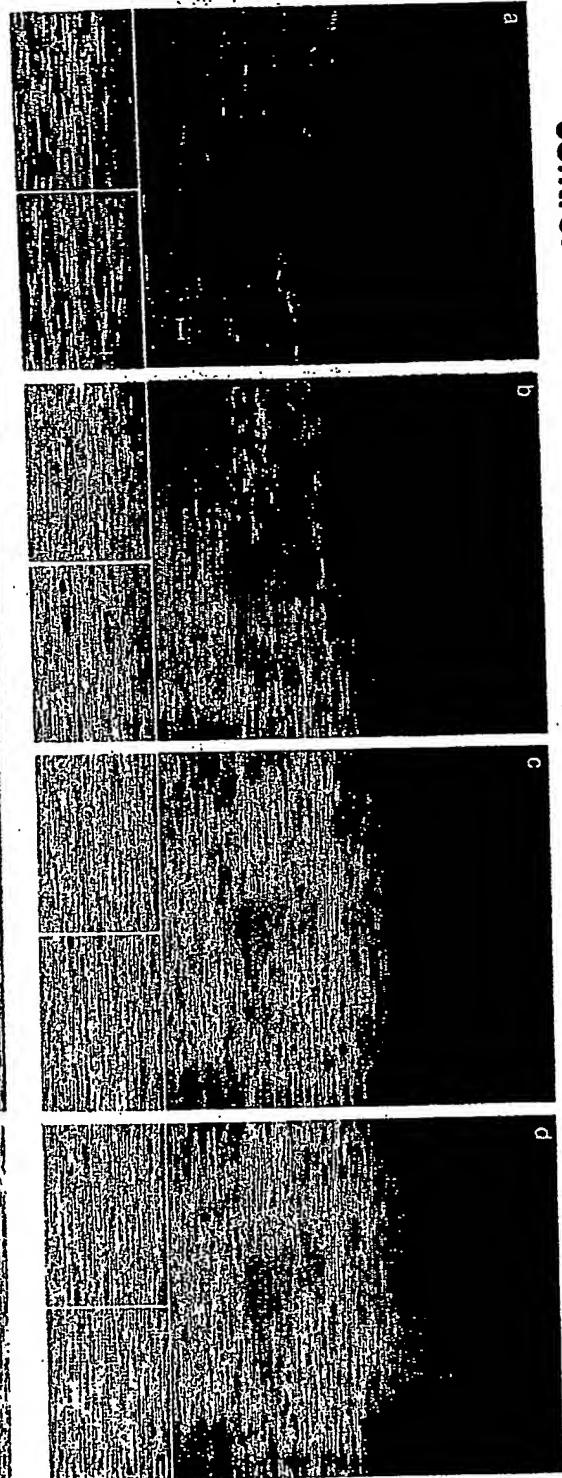


**a****b****Figure 2**

BrUTP



hrp 45



control      actinomycin D      65-2CTD injections      control injections

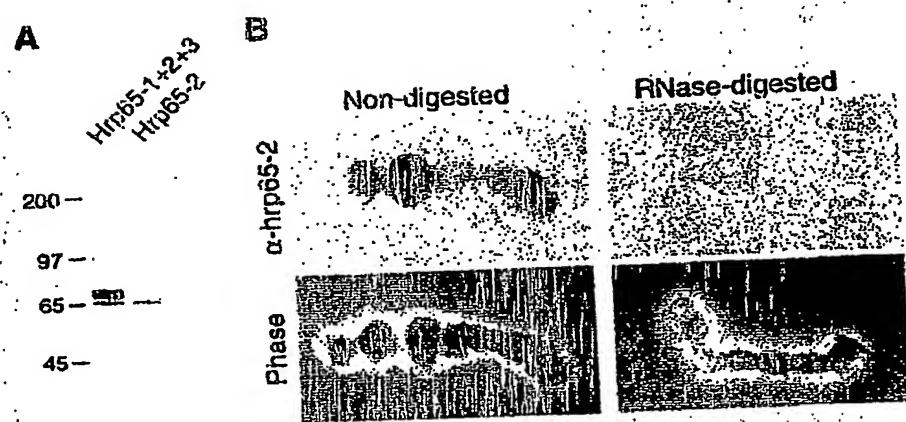


Figure 4

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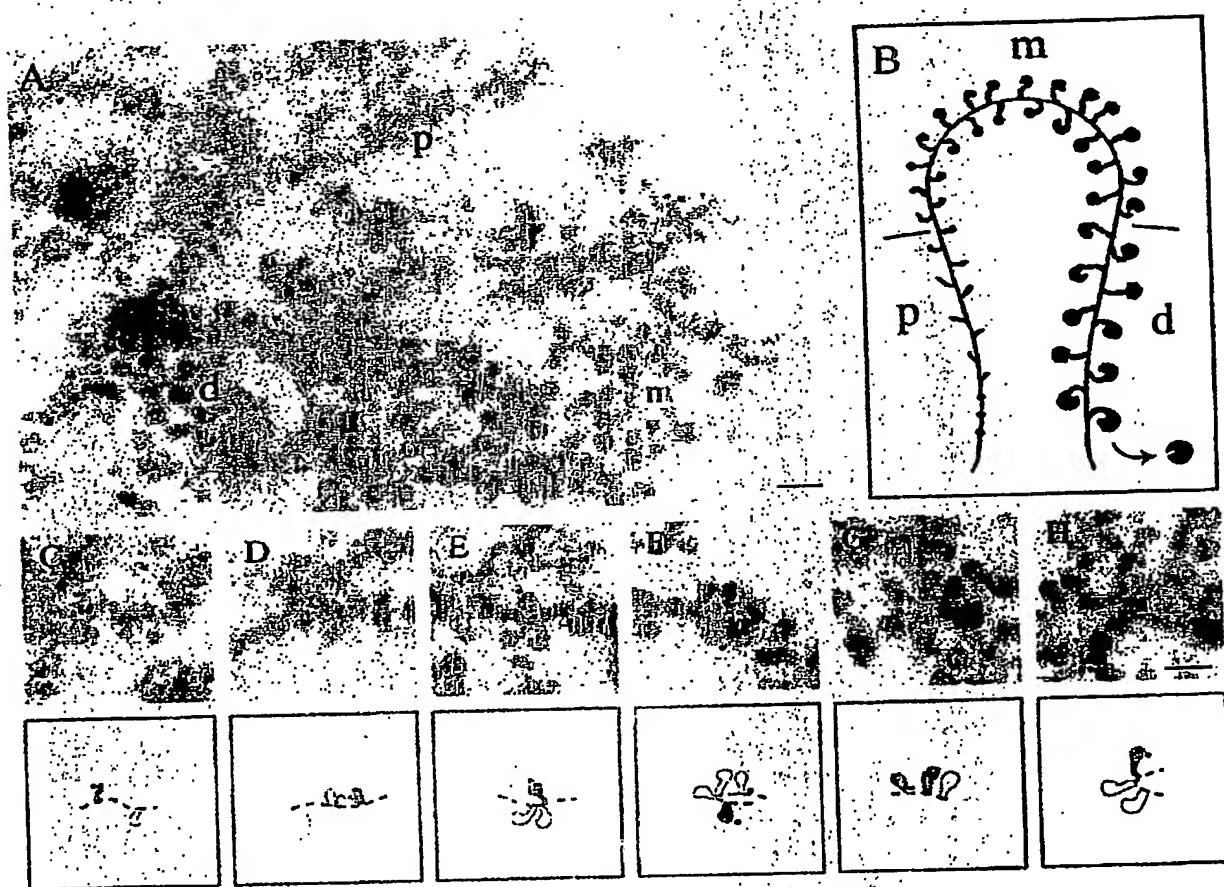


Figure 5

Inhibition of polyA+ RNA in mouse  
erythroleukemia cell lines

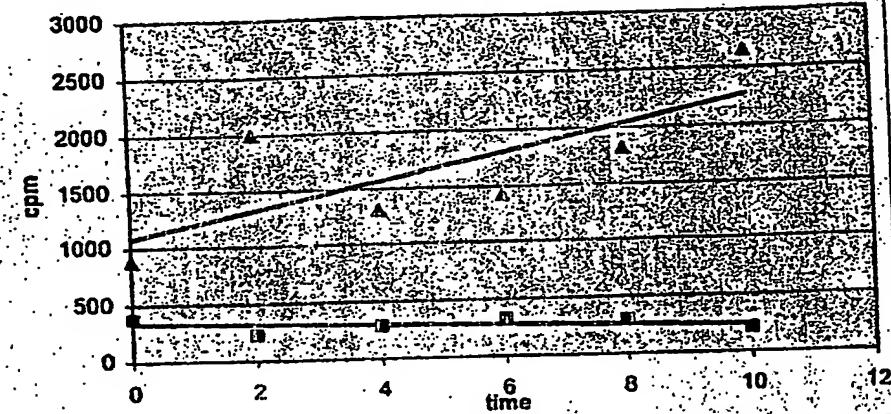


Figure 6

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